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Research article

Allergenicity assessment of *Delonix regia* pollen grain and identification of allergens by immunoproteomic approach

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ABSTRACT

Background: Plantation of road-side avenue trees has become a major part of urbanization programme for megacity beautification and environmental management. Due to evergreen habit and vibrant flower colour, *Delonix regia* (Gulmohor/Flamboyant) is frequently selected as ornamental tree for plantation programme. However, *D. regia* pollen is related to IgE mediated allergic reactions and no allergen has been reported so far.
Objective: Measuring the prevalence of *D. regia* pollen sensitivity among the local atopic individuals and identifying the allergens using immunoproteomic tools.
Methods: Aerobiological study was conducted for a period of two years to record the *D. regia* pollen concentration in the outdoor ambient air. Clinico-immunological tests were performed on atopic individuals to check the prevalence of sensitivity against *D. regia* pollen. Allergens were detected in the pollen proteome, fractionated in 1D and 2D gel by IgE serology and finally identified by mass spectrometry.
Result: In the study area *D. regia* pollen grains were present in ample amount in the air during May to July. About 38% of atopic individuals displayed positive Skin Prick Test (SPT) against *D. regia* pollen along with elevated level of specific IgE and histamine in the sera. Immunoproteomic analyses revealed the presence of 14 IgE reactive proteins in the 2D pollen proteome, of which 8 IgE reactive proteins were identified by MALDI TOF/TOF using homology driven proteomic approach.
Conclusion: This study demonstrated pollen related allergy symptoms by *D. regia* and gave significant message regarding the plantation programme to avoid the unnecessary load of allergic pollen. Also, a panel of 8 allergens were identified for the first time from *D. regia* pollen. Detailed study of these allergens would help to design immunotherapeutic strategies for pollinosis management.

1. Introduction

Allergic diseases such as allergic rhinitis, hay fever, allergic asthma, skin irritation, and anaphylaxis have become a major health problem, affecting the quality of life of millions of people worldwide. More than 300 million people all over the world is affected by different types of allergic diseases (Singh and Mathur, 2012; Pawankar, 2014). Pollen mediated allergic reactions have been found to be the most frequent type I allergies affecting up to 30% of the urbanized population (Pablos et al., 2016). Till now, more than 150 pollen allergens originating from weeds, grasses and trees have been officially acknowledged by the IUIS allergen nomenclature subcommittee (http://www.allergen.org/), (Xie et al., 2019). However, majority of them were reported from western countries. The incidence of allergic outbreak is also increasing in Asian countries. More than 25% of the Indian population is suffering from various types of allergic diseases (Bhattacharya et al., 2018). As vegetation of this area is entirely different from western countries, the sensitizing allergens are also varying. Only a few biochemical works was done previously on the identification of the offending pollen allergens of this region. So, there is an increasing need of molecular characterization of allergens of indigenous vegetation for developing proper treatment strategy in future (Bhattacharya et al., 2018).

Fabaceae is the third-largest family of flowering plants. In IUIS database, 3 plants of this family were reported to cause respiratory

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allergy and many others were reported to cause food mediated allergy (http://www.allergen.org/). IgE cross reactivity with other similar allergens of pollen or food origin was also detected in many of those reported allergens (Mittag et al., 2004a; Mittag et al., 2004b; Mittag et al., 2005; Peeters et al., 2007; Jappe and Vieths, 2010; Bar-El Dadon et al., 2014; Carlson and Coop, 2019; Bastiaan-Net et al., 2020). *Delonix regia* or Gulmohor (also nown as Flamboyant) is a member of this family, which is one of the most widely planted ornamental plants in the world. It is used as ornamental tree for megacity beautification and environmental management due to its evergreen nature and vibrant flower colour. Flowering of *D. regia* starts from end of March and continues till mid September. It is a very common tree found in Indian villages and also used in Urban Planning programmes to create Urban Green Spaces (UGS). Beside Indian sub-continent, it is widely planted in different parts of Southeast Asia, Northern Australia, Mediterranean parts of Europe, Africa, North America, and South America. Though ornamental plants bring benefits for human health and also help to reduce pollution, they can also be a source of allergenic pollen and thus can elicit respiratory hazards (Ribeiro et al., 2009; Bosch-Cano et al., 2011). Increased incidence of pollinosis was observed concomitantly with the increased use of such allergy causing ornamental plants (Cariñanos and Casares-Porcel, 2011; Charpin et al., 2019). Several immunoproteomic studies were done on several ornamental plants to identify the responsible allergens (Mousavi et al., 2017, 2019). Previous studies showed that *D. regia* pollen is an important source of aeroallergen, triggering respiratory allergies in atopic individual (Mandal et al., 2006, 2008) but detailed immuno-biochemical studies were lacking. Our study, to best of our knowledge, is the first detailed study to identify the allergens of *D. regia* pollen using clinical, immuno-biochemical and mass spectrometry based proteomic approaches.

In the present study, a two-year aerobiological sampling and clinical survey were conducted in Madhyamgram (about 19 km north of central Kolkata, West Bengal, India) to quantify the content of *D. regia* pollen grain in the air and its effect on atopic individuals. Allergenicity of *D. regia* pollen was first checked by Skin Prick Test (SPT) and further confirmed by in vitro tests, such as ELISA, histamine release assay. This was followed by detailed immuno-biochemical and proteomic approaches, which included pollen proteome profiling by SDS-PAGE and 2D-PAGE, IgE antibody specific reactive protein detection by immunoblots and finally identification of immuno-reactive proteins by mass spectrometry.

2. Materials and methods

2.1. Aerobiological study and collection of meteorological data

A continuous air sampling was carried out in Madhyamgram, West Bengal, India, a densely populated suburban area, from July 2015 to June 2017, using a seven-day Burkard automatic volumetric sampler (Burkard Manufacturing Co. Ltd., Hertfordshire, UK). The exposed tapes were mounted and microscopically examined according to the guidebook of The British Aerobiology Federation (1995). For pollen identification, several keys, atlases and pictures were followed. The pollen grains were counted in light microscope under 400X magnification and daily pollen load in per cubic meter of air was calculated. Meteorological parameters like temperature, wind speed, rainfall, and relative humidity and relative humidity were recorded from worldweatheronline.com to analyse their effects on pollen count in the air.

2.2. Pollen collection

Pure pollen grains of *D. regia* were collected from mature anthers of fresh flowers collected from the city during its flowering period. The pollen grains were then dried and grinded. Two hundred micron mesh was used to filter out unwanted debris. The material used throughout this work contained less than 1% of non pollen contaminants.

2.3. Preparation of crude antigenic extract of D. regia pollen

After defatting with diethyl ether, *D. regia* pollen grains were crushed in liquid nitrogen; 1 g pollen was mixed with 5 mL of sterile 0.1 M Phosphate buffer (PB), pH 7.2 (14 mL 0.2 M NaH_2PO_4, 36 mL 0.2 M Na_2HPO_4 for 100 mL 0.1 M PB, pH 7.2) and incubated overnight with constant stirring at 4 °C. The supernatant was collected after centrifugation at 12,000 × g for 20 min. The clear antigenic extract was obtained by filtering the supernatant through a 0.22 μm membrane (Merck Millipore, Darmstadt, Germany) and stored at -20 °C for further use.

2.4. Clinical study and collection of patient sera

SPT was performed on 150 allergic patients visiting to an allergy clinic with *D. regia* pollen extract. Smokers, pregnant women and patients with chronic diseases were excluded from the study. Clinical and demographic data of those patients were collected and then analyzed. The antigenic extract was diluted in PB (1:10 w/v) and 20 μL of diluted antigen solution was placed on the ventral side of the forearm and then pricked with a sterile lancet without inducing bleeding. Histamine diphosphate (1 mg/mL) and PBS (0.01 M, pH 7.2) were used as positive and negative controls, respectively. The wheal reaction was monitored after 20 min and graded according to Platts-Mills et al. (1981). Wheal diameter ≥3 mm was regarded as a positive response. Total IgE in the selected patients' sera was measured by the clinic and data was collected. Among these patients, 42 patients gave their written consent to use their blood in research purpose. Residual sera from these patients with positive cutaneous reaction to *D. regia* and elevated total IgE level were collected and then stored at - 20 °C for further immunological studies. Sera of six non-atopic individuals were also collected for negative control. Total free histamine content of these samples was measured using EIA Histamine kit (Immunotech, Marseille, France) following manufacturer's protocol. The entire study was approved by the Ethical committee of the institute (BIHEC/2014-15/4).

2.5. Specific IgE estimation

To quantify the specific IgE level in patients' sera against *D. regia* pollen extract, indirect ELISA was performed (Ghosh et al., 2015). Wells of microtiter ELISA plates (Nunc, Thermo Fisher, Waltham, Massachusetts,USA) were coated with crude antigenic extract (10 ng/μL) of *D. regia* pollen (50 μL/well) and incubated overnight at 4 °C. After repeated washing with 0.1 M PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T) and blocking with 3% Bovine Serum Albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) for 2h at 37 °C, wells were incubated with 50 μL of 1:10 diluted individual patient's sera at 4 °C for 16 h. Monoclonal anti-human IgE-alkaline phosphatase conjugate produced in mouse (Sigma-Aldrich) was used as secondary antibody and para –Nitro phenylphosphate (pNPP) (Sigma-Aldrich) was used as substrate. The reaction was stopped by adding 3 N NaOH and absorbance was measured at 405 nm in ELISA reader (Multiskan, Thermo Fisher).

2.6. Pollen protein extraction for proteome analysis

Total pollen protein of *D. regia* was extracted using trichloro-acetic acid (TCA)-acetone protocol. Around 100 mg of *D. regia* pollen was crushed in liquid N_2 and re-suspended in 4 mL of chilled acetone containing 10% TCA, 1% dithiothreitol (DTT) and 10 μL/mL protease inhibitor cocktail (Sigma-Aldrich), and incubated overnight at 4 °C. After centrifugation at 15000 × g at 4 °C for 30 min the pellet was collected and washed thrice with ice-cold acetone with 1% DTT. The air-dried pellet was re-suspended in 100 μL rehydration buffer (IEF) containing 7 M urea, 2 M thiourea and 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and kept at -20 °C for 16 h. Finally, it was centrifuged at 20,000 × g at 4 °C for 30 min; the supernatant was collected and stored at -80 °C for further studies. The

protein concentration of the extract was quantified using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Pollen protein profiling by 1D SDS-PAGE

Approximately 100 μg of *D. regia* pollen protein extract was mixed with 20 μL of sodium dodecyl sulphate (SDS) loading buffer (0.125 M Tris-HCl pH 6.8, 5% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue), boiled for 5 min and then resolved in 12% SDS-PAGE using mini vertical gel electrophoresis apparatus (GE-Healthcare, Chicago, Illinois) along with protein molecular weight marker (GeNei, Bengaluru, India). The protein profile was visualized by CBB – R 250 staining.

2.8. IgE specific western blot analysis

Specific IgE immunoblotting was carried out to detect the allergenic components in crude pollen protein extract (Sircar et al., 2012). *D. regia* pollen proteome resolved in 12% SDS PAGE was transferred onto polyvinylidene difluoride (PVDF) membrane (Merck) by semi-dry transfer method. The transfer was verified by staining with 0.1% Ponceau S (w/v) in 5% acetic acid. The membrane was then cut along each lane (10 mm strips), and each strip was washed properly with deionised water to remove Ponceau stain. Membrane strips were then blocked with 3% BSA in Tris buffer saline with 0.05% Tween-20 (TBS-T) (Bio-Bharati, Kolkata, India) for 1 h at 37 °C followed by washing with TBS-T. Primary antibody incubation was done overnight at 4 °C using individual patient's sera in 1:10 dilution in blocking solution. Seventeen individual patient sera with an elevated level of specific IgE (confirmed by ELISA) were used as the primary antibody, and three healthy sera were used as negative controls. Next day after washing, strips were incubated with secondary antibody, i.e., monoclonal anti-human IgE alkaline phosphatase conjugate (Sigma-Aldrich), in 1:1000 dilution for 3 h at 4 °C. IgE reactive bands were developed by addition of nitro-blue tetrazolium -5-bromo-4--chloro-3′-indolyphosphate (NBT-BCIP) (Sigma-Aldrich). The reaction was stopped by adding 0.5 M EDTA.

2.9. 2D gel electrophoresis

D. regia pollen protein extract of around 400 μg was processed using Focus Perfect 2D Clean up Kit (G Biosciences, St. Louis, MO, USA) following manufacturer's protocol and then reconstituted in 125 μL rehydration buffer (IEF) containing 1% immobilized pH gradient (IPG) buffer for pH 4–7 (v/v) (GE-Healthcare), 25 mM DTT and traces of Bromophenol blue. The sample was applied to 7 cm Immobiline Dry Strip (IPG strip, pH 4–7 L) (GE-Healthcare) in a re-swelling tray and left overnight at room temperature. Isoelectric focusing was performed next day using Ettan IPG phor-3 isoelectric focusing system (GE-Healthcare) as per manufacturer's protocol. Briefly, the strip was focused at 0–200 V for 1 min, 200–3500 V for 1.30 h and 3500 V for 1.15 h, with a total of 8 kV accumulated. Next day the strip was equilibrated with equilibration buffer – I (6 M Urea, 75 mM Tris-Cl pH 8.8, 30% glycerol, 2% SDS and 1% w/v DTT) for 15 min followed by equilibration buffer-II (same as equilibration buffer-I with 2.5% w/v iodoacetamide instead of DTT). Finally, the strip was run in 12% SDS-PAGE with mini vertical Electrophoresis System (GE-Healthcare). The gel was then stained with Coomassie Brilliant Blue- G250 and photographed in a Bio-Rad Versa Doc (Bio-Rad Laboratories) system.

2.10. 2D immunoblot

D. regia pollen proteins from 7 cm 2D gel were transferred onto PVDF membrane by semi-dry transfer method as described earlier in specific IgE immunoblot. After transfer, the membrane was blocked with 3% BSA and immunoblotted with pooled sera from 17 atopic patients. Another blot was done using pooled sera from healthy subjects as a negative control. Images of the blots were acquired in Bio-Rad Versa Doc (Bio-Rad Laboratories) system, and molecular weights of reactive spots were determined by Quantity One software (version 4.6.3, Bio-Rad Laboratories). Sero reactive spots with high signal (intensity value greater than 2) were considered for further proteomic analysis.

2.11. Sample preparation for mass spectrometry

For mass spectrometry (MALDI TOF/TOF), protein spots from CBB-stained 2D gel corresponding to 2D immunoblot were excised, cut into small pieces and subjected to in-gel trypsin digestion following the protocol described by Shevchenko et al. (2006) with slight modifications. Briefly, excised spots were first destained with 50 mM Ammonium Bicarbonate (pH 8.0) with 50% ethanol and then dehydrated by ACN. Following subsequent reduction and alkylation with 10 mM DTT and 55 mM Iodoacetamide respectively, spots were dehydrated again with ACN. In-gel digestion was carried at 37 °C with 12.5 ng/μL modified sequencing grade Trypsin Gold (Promega, Madison, WI, USA) for 16 h. Peptide fragments were eluted from gel pieces with extraction buffer containing 3% TFA and 30% ACN. Further extraction was done by dehydrating the gel pieces with 100% ACN. The final volume of the sample was reduced up to 10 times in Speed Vac (Thermo Fisher). Approximately, 1.5 μL of peptide digests were mixed with 5 volumes of 0.5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Bruker Daltonics, Hamburg Germany) spotted on MTP 384 ground steel target plate (Bruker Daltonics) and air-dried.

2.12. MALDI-TOF/TOF analysis

Mass spectra of trypsin digested proteins were obtained in Autoflex speed MALDI-TOF/TOF (Bruker Daltonics) under reflectron mode equipped with a pulsed nitrogen laser (l = 337 nm, 50 Hz) at 54% power in the positive ion mode. MS/MS was performed with top five tryptic peptides with high intensity and suitability (i.e., signal to noise ratio S/N > 20 and relative isolation) for fragmentation by Laser-Induced Dissociation (LID). MS/MS spectra were acquired by laser shots with a minimum of 4000 and a maximum of 8000 using the instrument calibration file. The spectra of parent and fragment ions were finally analyzed using SNAP algorithm using FlexAnalysis software (version 3.0, Bruker Daltonics).

2.13. Database search and allergen identification

MS/MS spectra were first processed using MS Biotools™ 3.4 (Bruker Daltonics) for inputting data into MASCOT search engine version 2.4.1 (MatrixScience.com) for protein identification. Specifications for MASCOT search include database: NCBInr; taxonomy: Viridiplantae, *Prosopis alba*; enzyme: trypsin; universal modification: cysteine carbamidomethylation; variable modification: methionine oxidation; missed cleavage: 1; mass tolerance of precursor ions: 0.5 Da; mass tolerance of fragment ions: 1.2 Da; peptide charge: +1. Since *D. regia* genome is unsequenced, spectral identification in MS was verified by MS/MS. The confidence level of protein identification was based on significant probability score and was considered reliable once a match of minimum 2 unique peptides or one unique peptide with extensive homology was obtained with a significance level of $p < 0.05$, which indicates 95% confidence level for a single protein. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD015697.

2.14. Statistical analysis

Calculation of Pearson's correlation co-efficient, p value and other statistical analysis were carried out in OriginPro software.

3. Result

3.1. Bio-monitoring estimated the aero-pollen load

Amount of air-borne pollen grains in the study area was determined by continuous air sampling for two years. Air-borne pollen grains are one of the main reasons of inhalative allergy. Some of the pollen grains which were present in high concentration in the air include pollen grains of *Trema orientalis,* Asteraceae, grasses, Chenopodiaceae-Amaranthaceae, *D. regia*, *Cocos nucifera, Alstonia scholaris, Areca catechu, Carica papaya* L. *etc.*

Tree pollen grains were found in higher amount than grass pollen grains throughout the year during the study period (Figure 1A). Aero-pollen load was lower during mid summer to monsoon in comparison to other seasons (Figure 1B). *D. regia* pollen grains were present in the air in ample amount at this time (Figure 1B). During its peak floweing time (May to July) *D. regia* pollen contributed major portion of aero-pollen load (Figure 1C).

3.2. Influence of weather parameters on D. regia pollen load

Effect of temperature, wind speed, precipitation, and relative humidity on pollen load was determined (Figure 1D). Wind speed was found to be significantly positively correlated ($r = 0.89$ at $p < 0.05$) with the amount of *D. regia* pollen grains in the air. Anemophilous mode of reproduction of *D. regia* increases the possibility of finding the pollen grains in the air.

3.3. Allergenicity assessment of D. regia pollen grains by skin prick test (SPT)

Skin prick test data indicated high allergy eliciting potential of *D. regia* pollen. Clinical details of the allergic patients selected for SPT are given in Supplementary table S1. Positive cutaneous response (SPT) to *D. regia* pollen grains was observed in 57 (38%) of the 150 patients tested (age range: 18–60). Most of the sensitive individuals showed +3 grade of positivity followed by +2 and +1 (Supplementary table S2). Positive skin reaction was not observed in healthy volunteers. Sex, age group and symptoms wise distribution of total allergic patients ($n = 150$) and *D. regia* allergic patients ($n = 57$) are shown in Figure 2A, B & C.

3.4. High level of total IgE and histamine content in D. regia pollen allergic patients

Elevated total IgE and total histamine levels were observed in the patients with *D. regia* pollen allergy as compared to healthy controls. The total IgE levels of healthy volunteers were below 100 IU/mL, whereas in patients it reached even up to 1776 IU/mL. The values of total histamine content in healthy volunteers were <20 nMol/L, but it was as high as 390 nMol/L in patients. There is a strong positive correlation (Pearson correlation coefficient $R = 0.7593$, $p < 0.00001$) exists between total histamine and total IgE in the patient sera (Figure 3A).

3.5. Estimation of serum specific IgE against D. regia pollen allergens

Specific IgE is the measure of allergenicity of a particular allergen in a patient. Specific IgE against *D. regia* pollen extract was measured in the

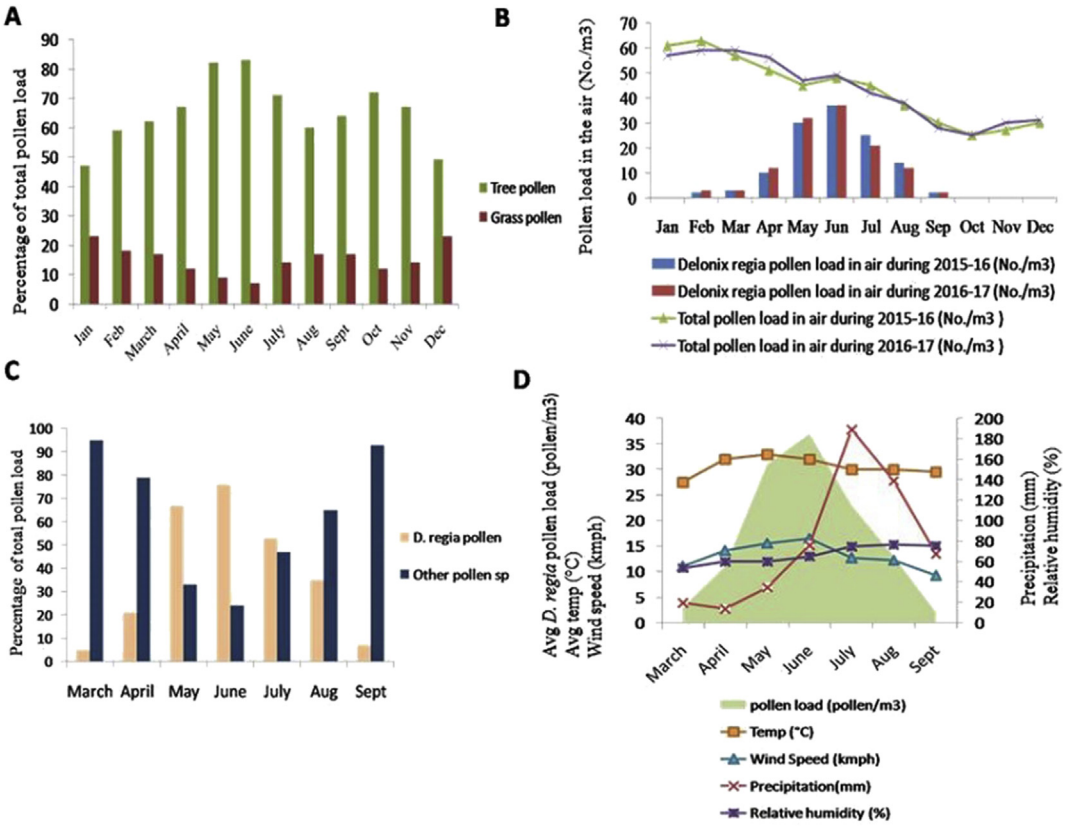

Figure 1. Air-borne pollen load and effect of different weather parameters on it. A. Comparison between tree pollen load and grass pollen load in the study area throughout the year during 2015–17. **B.** Comparative account of average number of total air-borne pollen load and *D. regia* pollen load per day represented in monthly basis during 2015–17; **C.** Percentage of total pollen load contributed by *D. regia* pollen and other pollen during the flowering period of *D. regia*. **D.** Analysis of *D. regia* pollen load during its flowering period and different weather parameters during this period.

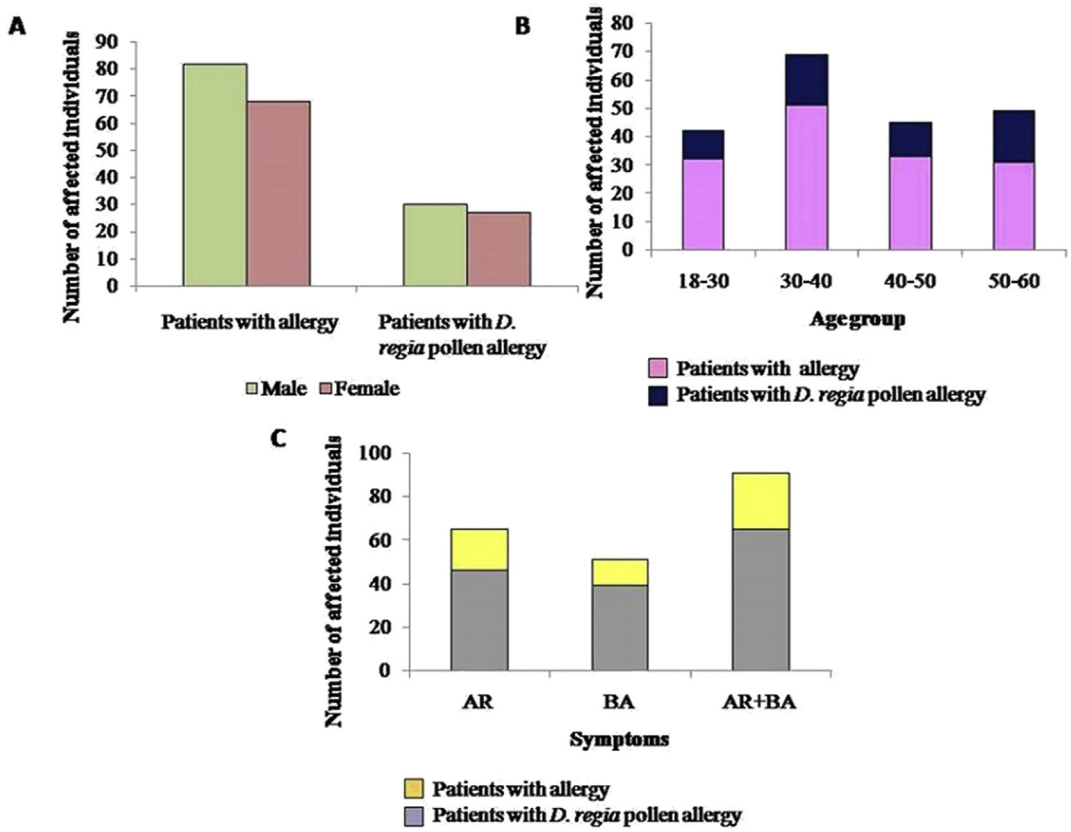

Figure 2. Demographic features of patients (n = 150). A. Sex ratio of allergy patients **(n = 150)** and *D. regia* pollen allergic patients (n = 57) represented in different panels; **B.** Age group wise distribution of patients suffering from allergy (n = 150) and *D. regia* pollen allergic patients (n = 57); **C.** Symptom wise distribution of patients with allergy (n = 150) and *D. regia* pollen allergy (n = 57). **AR-** Allergic Rhinitis, **BA-** Bronchial Asthma.

collected sera of 42 patients and 6 healthy individuals. Details of these patients are given in Table 1. Many of the allergic patients showed concomitant allergy with other pollen grains and fungal allergens. Average $O.D._{405}$ value of healthy individuals was 0.11. All the atopic patients showed more than 2-fold increase in the $O.D._{405}$ value with respect to healthy sera ($O.D._{405}$ value >0.22) and regarded as sensitized by *D. regia* pollen. The specific IgE value of the patients showed strong positive correlation with total IgE (Pearson correlation coefficient $R = 0.7946$, $p < 0.00001$) (Figure 3B) and total histamine (Pearson correlation coefficient $R = 0.833$, $p < 0.00001$) (Figure 3C) level of them.

3.6. Total protein profiling by gel electrophoresis

Total protein of *D. regia* pollen was fractionated in 12% SDS-PAGE revealing more than thirty bands within a molecular weight ranged between 14 and 97 kDa (Figure 4A). For detailed proteome profiling, two-dimensional gel electrophoresis was a useful way, where proteins were separated according to their isoelectric point (pI) and molecular weight (MW). Different proteins with same molecular weight which could not be identified separately in the 1D gel can easily be separated in 2D gel electrophoresis. Protein bands of *D. regia* were resolved in more than hundred spots within isoelectric point (pI) range of 4–7 horizontally and MW range of 14.3–97.4 kDa vertically as illustrated in Figure 4B.

3.7. Detection of allergens by 1D and 2D immunoblot

The IgE binding proteins were detected by confronting the SDS PAGE separated proteins of *D. regia* with seventeen individual patient sera (Figure 5A, Lane 1–17). The immunoblots showed six distinct seroreactive bands at MW of 23 kDa, 30 kDa, 35 kDa, 42 kDa, 44 kDa and 70 kDa. Frequency of these immunoreactive bands in those 17 atopic individuals is graphically presented in Figure 5B. The 30 kDa immunoreactive protein band was found in more than 90% of the total patients' sera (n = 17) screened by immunoblotting and hence can be considered as predominant allergen (Figure 5B). Immunoblots with non-atopic healthy individuals are represented as negative controls in Figure 5A, Lane C1–C3. Pooled sera of 17 patient was used to detect IgE reactive spots in 2D immunoblot and revealed 14 IgE reactive spots of MW 16, 18, 30, 31, 42, 52, 55, 60, 64, 67, 68, 70 kDa as shown inFigure 6A & B.

3.8. Mass spectrometry based identification of allergens

Eight IgE reactive proteins of *D. regia* pollen were identified by mass spectrometry (Table 2). As *D. regia* genome is unsequenced, the ion spectra of proteins generated in MALDI TOF/TOF were searched against NCBInr database for homology searching against Viridiplantae and *Prosopis alba*. Seven Immuno-reactive spots matched with minimum two unique peptides of specific protein. These proteins were Luminal-binding 5 (spot number 2), ATP synthase beta subunit (spot number 5), Actin (spot number 6, 7), ADP, ATP carrier protein 1 (spot no 8), Recombinant S-adenosylmethionine synthase 2 (spot number 9) and UDP-arabinopyranose mutase (spot number 13). In case of spot no 11, only one unique peptide match was found with hypothetical protein OsJ_04810 with significant score. No homology matching was found for the remaining 6 spots and therefore remained unidentified.

4. Discussion

Respiratory allergic diseases have been increasing at an alarming rate worldwide with India being not an exception; however, the major drawback of developing efficient treatment strategy in the Indian subcontinent is the lack of knowledge about the offending allergens. In

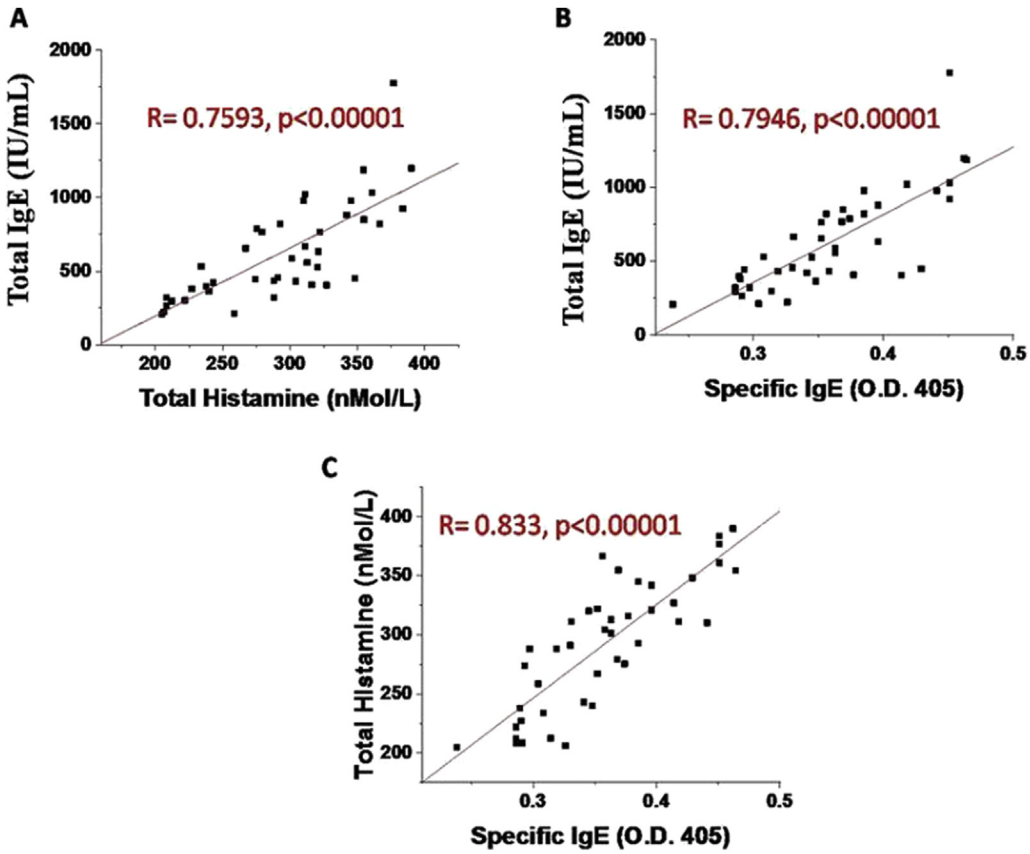

Figure 3. Correlation curve of different allergy parameters. A. Correlation between total IgE and total histamine values, **B.** total IgE and specific IgE values, **C.** total histamine and specific IgE values of *D. regia* pollen allergy patients showing significant relatedness between them.

this study a continuous aerobiological monitoring was done from 2015-17 to know the aero-pollen load of the study area. *D. regia* pollen grains were present in considerable amount in the air from May to July, which is in accordance with earlier reports (Mandal et al., 2006, 2008) except the peak flowering month that has shifted slightly due to changed environmental conditions. This tree has been increasingly used in urban areas for creating green space which has concomitantly uplifted the *D. regia* pollen load in the air. However, no previous report was found on detailed immunoproteomic characterization of the *D. regia* pollen allergens.

Allergenicity of *D. regia* pollen was tested by several clinical and immuno-biochemical studies and 38% of atopic individuals were found to be allergic to *D. regia* pollen. According to the previous report (Mandal et al., 2008) lesser percentage of allergic patients were found to be SPT positive against the same pollen grain. Epidemiologic studies have shown that climate change, urbanization, high levels of vehicle emissions, and westernized lifestyle are correlated to an increase in the prevalence of pollen induced respiratory allergy (D'amato, 2000; Ariano et al., 2010; Bielory et al., 2012; Barnes, 2018). Greenhouse gasses and total pollutant not only increase the pollen production but it also increase allergenicity (Beck et al., 2013). Rapid urbanization and climate change in past few years might be accountable for the upsurge of allergic potency of *D. regia* pollen. Immunoproteomic studies detected 14 IgE reactive proteins of which 8 allergens were identified by mass spectrometry. A major drawback in mass spectrometry based identification is that it largely depends on the presence of complete protein database and thus determining correct proteins from unsequenced species like *D. regia* was challenging. In spite of these constrains majority of the IgE reactive spots were identified by homology driven proteomics. Spot number 2 of 2D immunoblot (Figure 6) showed homology with Luminal-binding 5 protein. Luminal binding proteins (BiP) belong to heat shock protein 70 (Hsp 70) family, which is a well-known pan-allergen (Gruehn et al., 2003). BiP functions as a chaperone during protein synthesis. IgE reactive property of BiP was reported previously along with its cross-reactive nature with other pollen allergens (Gruehn et al., 2003; Nayak et al., 2013). It was reported as Cor a 10 in WHO/IUIS database from European hazel (Gruehn et al., 2003). Spot number 5 was identified as ATP synthase beta subunit, which is a mitochondrial membrane-bound protein, essential for the synthesis of ATP. This protein was already identified as allergic protein in *Cannabis sativa* (Nayak et al., 2013), *Ligustrum lucidum* (Mani et al., 2015), *Lantana camara* (Ghosal et al., 2016) and *Quercus rubra* (Huerta-Ocampo et al., 2020). It has not been enlisted in WHO/IUIS database yet. Spot no 6 was identified as actin, which is an essential component of cell cytoskeleton and participates in many important cellular processes, including cell motility, cell division, various plant cell signalling events etc. Actin was earlier reported as a major allergen from molluscs (Mohamad-Yadzir et al., 2015; Azofra et al., 2017; González-Mancebo et al., 2019), which is recently reported as a cross-reactive allergen (Barbarroja-Escudero et al., 2019). However, no report was available on the allergenic nature of this protein of plant origin. Spot number 8 showed homology with ADP, ATP carrier protein 1. This protein is involved in ATP transmembrane transporter activity. No earlier report exists stating the allergenic nature of this protein. Spot number 9 was identified as S-adenosylmethionine synthase, which catalyzes the formation of S-adenosylmethionine from methionine and ATP. Allergenic nature of this protein was earlier reported from *Cocos nucifera* (Saha et al., 2015). It has not been enlisted in WHO/IUIS database yet. Spot number 11 showed homology with a hypothetical protein OsJ_04810 which functions as an E3 ubiquitin ligase. Spot number 13 was identified as UDP-arabinopyranose mutase, involves in the biosynthesis of cell wall non-cellulosic polysaccharides. None of them were previously reported as allergen.

Table 1. Demographic and Clinical details of the patients with *Delonix regia* pollen allergy enrolled in the study.

Patient Number	Age	Sex	Concomitant allergy to other allergen	Symptoms*	SPT to *Delonix regia* (>3 mm)**	Total IgE(IU/mL)	Specific IgE (O.D. value at 405 nm)	Total Histamine content (nMol/L)	IgE reactive protein bands (kDa)
1	31	F	Pollen- Yes Fungus- No	BA	+2	265	0.291	208	
2	18	M	Pollen- Yes Fungus- Yes	AR + BA	+3	405	0.414	327	30, 70
3	34	F	Pollen- Yes Fungus- Yes	AR + BA	+3	450	0.429	348	30, 35, 42, 44, 70
4	54	M	Pollen- Yes Fungus- No	AR	+3	408	0.377	316	30
5	57	F	Pollen- No Fungus- Yes	AR + BA	+3	1187	0.464	354	23, 30, 35, 42, 44, 70
6	20	F	Pollen- No Fungus- No	BA	+2	223	0.326	206	
7	58	F	Pollen- Yes Fungus- Yes	AR + BA	+3	922	0.451	383	30, 35, 42
8	34	M	Pollen- Yes Fungus- Yes	BA	+2	213	0.304	258	
9	37	F	Pollen- Yes Fungus- No	BA	+2	208	0.238	204	
10	45	F	Pollen- Yes Fungus- Yes	AR	+3	432	0.358	304	
11	49	M	Pollen- Yes Fungus- Yes	AR	+3	528	0.345	320	
12	35	M	Pollen- No Fungus- Yes	AR	+2	381	0.290	227	
13	51	M	Pollen- Yes Fungus- Yes	AR + BA	+3	849	0.369	354	
14	55	M	Pollen- Yes Fungus- Yes	AR + BA	+3	820	0.356	366	
15	30	F	Pollen- Yes Fungus- Yes	AR + BA	+3	365	0.348	239	
16	19	F	Pollen- Yes Fungus- Yes	AR	+2	298	0.314	212	
17	49	F	Pollen- Yes Fungus- Yes	AR + BA	+3	978	0.441	310	30
18	32	M	Pollen- Yes Fungus- Yes	AR + BA	+3	421	0.341	243	
19	20	F	Pollen- Yes Fungus- Yes	AR	+3	587	0.363	301	30
20	21	M	Pollen- Yes Fungus- Yes	AR + BA	+3	632	0.396	321	30
21	35	M	Pollen- Yes Fungus- Yes	AR + BA	+3	1032	0.451	361	30, 35
22	37	M	Pollen- Yes Fungus- No	AR	+2	321	0.286	208	
23	60	F	Pollen- No Fungus- Yes	AR	+3	531	0.308	234	
24	57	F	Pollen- Yes Fungus- Yes	AR + BA	+3	1776	0.451	376	30, 42, 44

(*continued on next page*)

Table 1 (*continued*)

Patient Number	Age	Sex	Concomitant allergy to other allergen	Symptoms*	SPT to *Delonix regia* (>3 mm)**	Total IgE(IU/mL)	Specific IgE (O.D. value at 405 nm)	Total Histamine content (nMol/L)	IgE reactive protein bands (kDa)
25	33	M	Pollen- Yes Fungus- Yes	BA	+2	433	0.319	287	
26	39	F	Pollen- Yes Fungus- No	BA	+2	456	0.330	291	
27	49	F	Pollen- Yes Fungus- Yes	AR	+3	558	0.363	312	44
28	47	M	Pollen- Yes Fungus- Yes	AR	+3	879	0.396	342	30
29	50	M	Pollen- Yes Fungus- Yes	AR + BA	+3	764	0.352	322	
30	51	M	Pollen- No Fungus- Yes	BA	+2	295	0.286	212	
31	59	F	Pollen- Yes Fungus- Yes	AR + BA	+3	788	0.374	275	
32	35	F	Pollen- Yes Fungus- Yes	AR + BA	+3	821	0.385	292	30
33	39	M	Pollen- Yes Fungus- Yes	AR	+3	655	0.352	267	
34	44	F	Pollen- Yes Fungus- No	AR	+2	302	0.286	221	
35	47	F	Pollen- Yes Fungus- Yes	AR + BA	+3	767	0.368	279	30
36	58	M	Pollen- Yes Fungus- Yes	AR + BA	+3	1021	0.418	311	23, 30, 35, 42, 44, 70
37	51	M	Pollen- No Fungus- Yes	BA	+2	321	0.297	288	
38	29	M	Pollen- Yes Fungus- No	AR	+2	445	0.293	274	
39	48	M	Pollen- Yes Fungus- Yes	AR	+2	398	0.289	237	
40	38	F	Pollen- Yes Fungus- No	BA	+3	667	0.331	311	
41	55	M	Pollen- Yes Fungus- Yes	AR + BA	+3	978	0.385	345	30, 35, 42
42	58	M	Pollen- Yes Fungus- Yes	AR + BA	+3	1198	0.462	390	23, 30, 35, 42, 44, 70
C1	47	M	Pollen- No Fungus- No	NS	-	40	0.119	18	
C2	36	M	Pollen- No Fungus- No	NS	-	67	0.106	13	
C3	19	F	Pollen- No Fungus- No	NS	-	55	0.112	12	
C4	27	F	Pollen- No Fungus- No	NS	-	30	0.109	10	
C5	50	M	Pollen- No Fungus- No	NS	-	45	0.114	16	
C6	30	F	Pollen- No Fungus- No	NS	-	50	0.104	12	

* Abbreviations: AR- Allergic Rhinitis, BA- Bronchial Asthma, NS- No Symptom, C1 –C6 –Non-atopic healthy subjects.

** The grading scale of positive skin prick reactions is as follows: negative if wheal diameter is < 3 mm, +1 if wheal diameter is 3–5 mm, +2 if wheal diameter is > 6 mm, +3 if wheal diameter is > 6 mm, with one or two small pseudopodes.

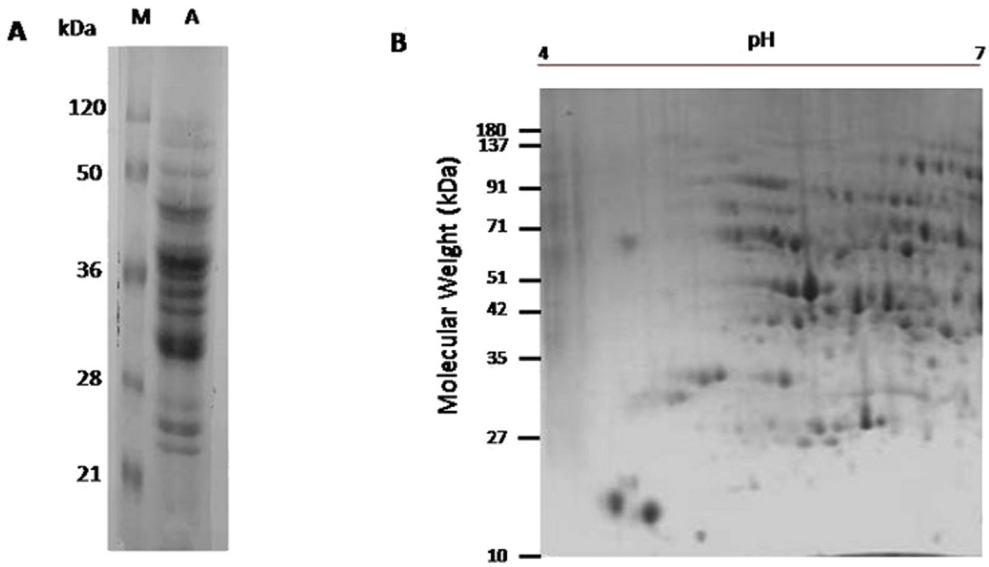

Figure 4. Total protein profiling of *D. regia* pollen grains in SDS PAGE (A) and 2D PAGE (B). **Lane M** = Molecular weight marker, **Lane A**= Total protein profile.

In a nutshell, our study identified eight allergens from *D. regia* pollen. Out of them, five were previously reported as allergens from different sources whereas the rest three are reported as novel *D. regia* allergen for the first time. The theoretical MW and PI values of some of the identified proteins were different from that of the observed values in the gel. Post-translational modifications, such as deamidation, phosphorylation and other charge modifying variations along the polypeptide chains should be the probable reason.

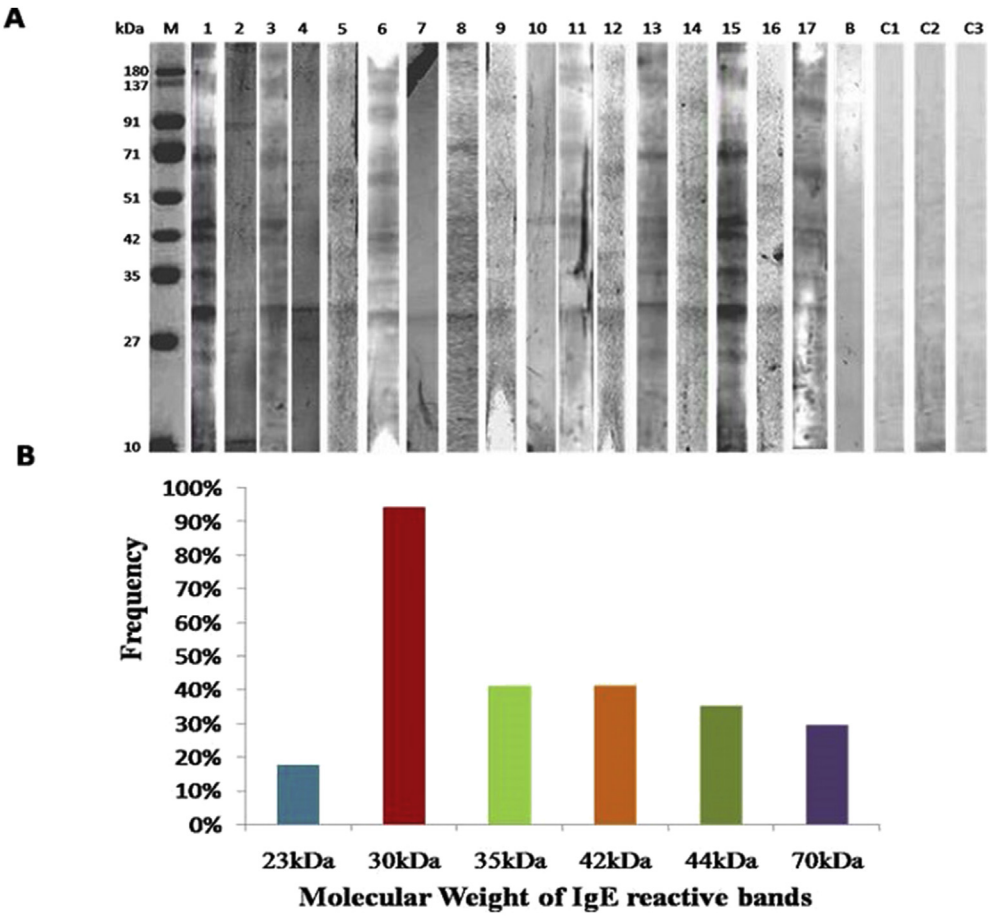

Figure 5. Immunoscreening of the allergens of *D. regia* crude pollen extract and sensitization frequency of these allergens in patient population. A. Western blot showing IgE reactive bands in 17 atopic patient sera. **Lane 1–17**= IgE specific immunoblots with individual serum of seventeen *D. regia* allergic patients. **Lane B**= Buffer control; **Lane C1–C3**= Negative control blots with individual serum of three healthy individuals. **B.** Percentage of sensitization prevalence of the IgE reactive bands among patient population.

Table 2. Mass spectrometry (MALDI-TOF/TOF) based identification of IgE reactive proteins of *D. regia* pollen.

Spot no.	NCBI accession no.	Protein name	No. of matched unique peptides	Sequence of unique peptides	Significance score (E value)	Thr. Mass (kDa)/pI	Gel mass (kDa)/pI	Significant score	Masot score of the matched peptide	Biological function
2	KHG04866.1	Luminal-binding 5 [*Gossypium arboreum*]	2	R.VEIESLFDGIDFSEPLTR.A	0.0051	81/5.46	70/5.8	55	65	Probably plays a role in facilitating the assembly of multimeric protein complexes inside the ER
	XP_028774695.1	luminal-binding protein 5 [*Prosopis alba*]		K.FDLSGIPPAPR.G	0.0011	73.5/5.14		33	50	
5	XP_028757835.1	ATP synthase subunit beta, mitochondrial-like [*Prosopis alba*]	4	K.VVDLLAPYQR.G	0.016	60/5.8	67/5.4	33	39	Mitochondrial membrane ATP synthase (F_1F_0 ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane. Subunits alpha and beta form the catalytic core in F_1. Rotation of the central stalk against the surrounding alpha$_3$beta$_3$ subunits leads to hydrolysis of ATP in three separate catalytic sites on the beta subunits.
	XP_028757835.1	ATP synthase subunit beta, mitochondrial-like [*Prosopis alba*]		R.VLNTGSPITVPVGR.A	0.0012	60/5.8		31	48	
	XP_028757835.1	ATP synthase subunit beta, mitochondrial-like [*Prosopis alba*]		R.IPSAVGYQPTLATDLGGLQER.I	3e-014	60/5.8		29	152	
	PNY00126.1	ATP synthase beta subunit [*Trifolium pratense*]		R.VGLTGLTVAEHFR.D	4.2e-06	36.9/8.4		53	94	
6	XP_028753182.1	actin-101-like [*Prosopis alba*]	4	R.AVFPSIVGRPR.H	0.00042	41.8/5.3	55/5.5	28	49	Highly conserved proteins involved in various types of cell motility, essential component of cell cytoskeleton; plays an important role in cell shape determination, cell division, cytoplasmic streaming, organelle movement and also involves in various plant cell signalling events.
	XP_028753182.1	actin-101-like [*Prosopis alba*]		K.SYELPDGQVITIGAER.F	5.4e-012	41.8/5.3		31	131	
	XP_028753182.1	actin-101-like [*Prosopis alba*]		K.DLYGNIVLSGGSTMFPGIADR.M	9e-014	41.8/5.3		30	148	
	XP_028753182.1	actin-101-like [*Prosopis alba*]		R.TTGIVLDSGDGVSHTVPIY EGYALPHAILR.L	5.2e-012	41.8/5.3		28	128	
7	XP_028775775.1	actin [*Prosopis alba]*	2	K.AEYDESGPSIVHR.K	1.6e-007	41.8/5.3		33	88	Actin
	XP_028775775.1	actin [*Prosopis alba]*		K.SYELPDGQVITIGAER.F	7.6e-013	41.8/5.31		32	140	
8	XP_014493651.1	ADP, ATP carrier protein 1, mitochondrial-like [*Vigna radiata var. radiata*]	2	R.LADDDEFMVPSVFNSR.F	0.017	41.7/9.7	42/5.4	52	57	ATP transmembrane transporter activity
	XP_028789657.1	ADP, ATP carrier protein 1, mitochondrial-like isoform X1 [*Prosopis alba*]		K.GNFLIDFLMGGVSAAVSK.T + Oxidation (M)	0.00018	43.5/9.7		32	57	
9	XP_028770998.1	S-adenosylmethionine synthase 1-like [*Prosopis alba*]	3	R.FVIGGPHGDAGLTGR.K	2.5e-009	43.5/5.57	60/6.9	32	106	Catalyzes the formation of S-adenosylmethionine from methionine and ATP.
	XP_028775227.1	S-adenosylmethionine synthase 1-like [*Prosopis alba*]		K.TAAYGHFGRDDPDFTWETVK.L	4.6e-005	43/6.08		31	62	
	XP_028775227.1	S-adenosylmethionine synthase 1-like [*Prosopis alba*]		K.TCPWLRPDGK.T	0.011	43/6.08		34	41	
11	EAZ14879.1	hypothetical protein OsJ_04810 [*Oryza sativa* Japonica Group]	1	R.EGEGEGGVAAAGVGVR.R	0.00037	33.9/10.8	30/5.4	56	78	Functions as an E3 ubiquitin ligase with specific E2 ubiquitin-conjugating enzymes. Undergoes auto-ubiquitination.
13	XP_006430944.1	UDP-arabinopyranose mutase 1 [*Citrus clementina*]	3	K.TINVPDGFDYELYNR.N	0.00062	42.5/5.5	16/4.7	55	75	UDP-L-arabinose mutase involves in the biosynthesis of cell wall non-cellulosic polysaccharides.
	PIN13397.1	UDP-arabinopyranose mutase [*Handroanthus impetiginosus*]		-.MSKPATPVAPP LKDELDIVIPTIR.N +	0.0084	41.8/6		53	62	
	XP_028755943.1	UDP-arabino pyranose mutase 1 [*Prosopis alba*]		K.ASNPFVNLR.K	0.0021	41/5.6		34	48	

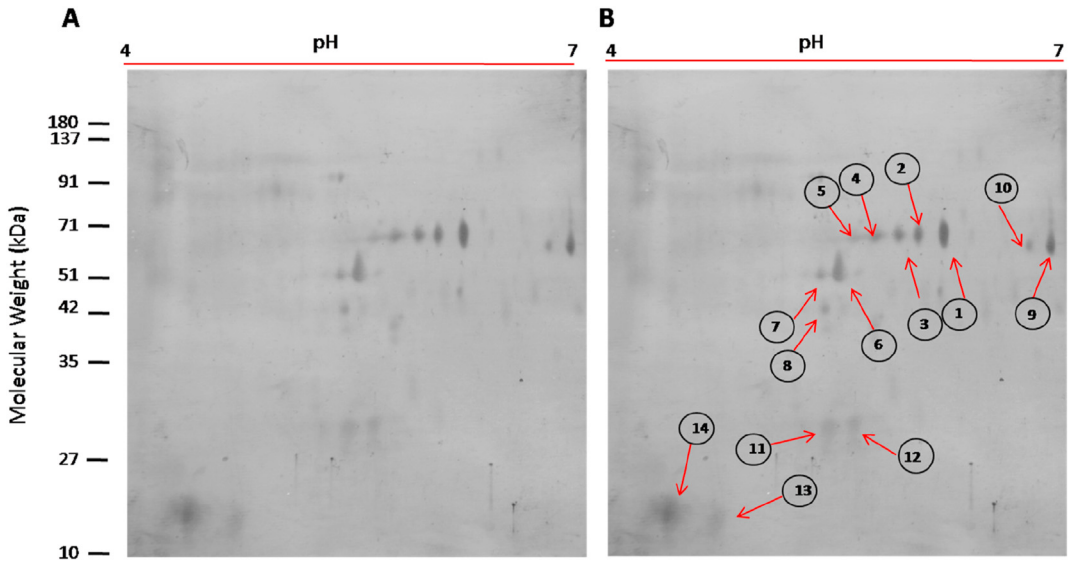

Figure 6. IgE specific 2D immunoblot with pooled sera from seventeen atopic individuals revealed fourteen distinct IgE reactive spots (A) which were marked in (B).

5. Conclusion

This study showed that *D. regia* pollen grain remains in considerable amount in the air during its peak flowering season (May to July) and can elicit IgE mediated allergic symptoms in atopic individuals. This result can be taken as an indicator of potential risk for pollen allergy sufferers and should be considered in future urban green area planning programme. Further studies such as purification, recombinant expression and immunological characterization of individual allergenic components will be beneficial to improve the component resolved diagnosis and immunotherapy of pollen allergy.

Declarations

Author contribution statement

Moumita Bhowmik: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nandini Ghosh: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Swati Gupta Bhattacharya: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at PRIDE Repository under Dataset identifier: PXD015697.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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Supplementary Table S1: Clinical Details of the 150 allergic patients

Patient Groups	Number of Patients
Gender	
Male	82
Female	68
Age	18-60
Diagnosis	
Allergic rhinitis	46
Asthma	39
Allergic rhinitis + Asthma	65
Age of Onset	
<20 years	64
≥20 years	86
Family history of allergic diseases	
Yes	70
No	80

Supplementary Table S2: SPT response grade in the study samples against *D. regia* pollen.

Skin Prick Test response	No of Individuals
-Ve	93
+1	8
+2	19
+3 or more	30